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Active hedgehog protein mutant, process for its production and use

The invention concerns an active form of a hedgehog protein, a process for its recombinant production and its therapeutic use. Hedgehog (hh) proteins are understood as a family of secreted signal proteins which are responsible for the formation of numerous structures in embryogenesis (J.C. Smith, Cell 76 (1994) 193 - 196, N. Perrimon, Cell 80 (1995) 517 - 520, C. Chiang et al., Nature 83 (1996) 407, M.J. Bitgood et al., Curr. Biol. 6 (1996) 296, A. Vortkamp et al., Science 273 (1996) 613, C.J. Lai et al., Development 121 (1995) 2349). During its biosynthesis a 20 kD N-terminal domain and a 25 kD C-terminal domain are obtained after cleavage of the signal sequence and autocatalytic cleavage. The N-terminal fragment is modified with lipid (J.A. Porter et al., Science 274 (1996) 255 - 259). In higher life-forms the hh family is composed of at least three members i.e. sonic, indian and desert hh (Shh, Ihh, Dhh; M. Fietz et al., Development (Suppl.) (1994) 43 - 51). Differences in the activity of hedgehog proteins that were produced recombinantly were observed after production in prokaryotes and eukaryotes (M. Hynes et al., Neuron 15 (1995) 35 - 44 and T. Nakamura et al., Biochem. Biophys. Res. Comm. 237 (1997) 465 - 469.

Hynes et al. compare the activity of hh in the supernatant of transformed human embryonic kidney 293 cells (eukaryotic hh) with hh produced from E. coli and find a four-fold higher activity of hh from the supernatants of the kidney cell line. A potential

additional accessory factor which is only expressed in eukaryotic cells, a post-translational modification, a different N-terminus since the hh isolated from E. coli contains 50 % of a hh which carries two additional N-terminal amino acids (Gly-Ser) or is shortened by 5 - 6 amino acids, or a higher state of aggregation (e.g. by binding to nickel agarose beads) have been discussed to be the reason for this increased activity.

Nakamura et al. compare the activity of shh in the supernatant of transformed chicken embryo fibroblasts with an shh fusion protein isolated from E. coli which still has an N-terminal polyhistidine part. The shh in the supernatant of the fibroblasts has a seven-fold higher activity than the purified E. coli protein with regard to stimulation of alkaline phosphatase (AP) in C3H10T ½ cells. Molecules such as bone morphogenetic proteins (BMPs) have been discussed as the reason for the increased activity which are only present in the supernatant of eukaryotic cells and cause the stronger induction of AP.

Kinto et al., FEBS Letters, 404 (1997) 319 - 323 describe that fibroblasts which secrete hh induce ectopic bone formation in an i.m. implantation on collagen.

The object of the invention is to produce hh proteins (polypeptide) which have a considerably improved activity compared to the known forms.

The object is achieved by a post-translationally processed hedgehog protein mutant (hh mutant) which is obtainable by expression of a gene which codes a

hedgehog protein in a baculovirus expression system in a fermentation over a period of up to 30 hours, preferably 24-27 hours, purification of the cell supernatant in the presence of a protease inhibitor and a non-ionic detergent and isolation of the hh mutant which binds to heparin-Sepharose and hydroxylapatite and is characterized in that this hh mutant

- exhibits a molecular weight of 22 ± 1 kDa under alkylating conditions,
- exhibits a molecular weight of 24 ± 1 kD under reducing conditions,
- is stabilized with respect to its activity by suramin
- is inactivated when 8 or more amino acids are cleaved N-terminally
- is inactivated by 90 % or more when incubated with 10 mmol/l 1.4 dithioerythritol (DTE) for 2.5 hours at 37°C,
- induces an activity for alkaline phosphatase of ca. 90 nmol pNP/min/mg at a concentration of 5 nmol/l in the presence of suramin,
- is not modified by cholesterol.

Activity within the sense of the invention is understood as the activity of alkaline phosphatase which the polypeptide can induce in mammalian cells (activity in the alkaline phosphatase test). In this method a mouse fibroblast cell line is cultured in a medium which contains foetal calf serum. Subsequently sterile filtered sample is added, the cells are lysed after ca. 5 days and alkaline phosphatase is determined in the cell lysate by means of the cleavage of a chromogenic substrate (pNP, p-nitrophenol) (J. Asahina, Exp. Cell. Res. 222 (1996) 38 - 47 and T. Nakamura (1997)).

A baculovirus expression system is understood as an expression system composed of a baculovirus vector and an insect cell as the host cell. Such expression systems are known to a person skilled in the art and are for example described by Bumcrot (1995) for hh proteins.

A hedgehog protein is understood by the invention as a secreted signal protein which is responsible for the formation of numerous structures in embryogenesis. Sonic, indian or desert hh are particularly preferably used (M. Fietz et al. (1994). A hh protein with a sequence as described in the EMBL database under the No. L38518 is preferably used. Proteins of the hedgehog family exhibit a pronounced homology in their amino acid sequence which is why it is also preferable to express those nucleic acids which code for hedgehog proteins which are 80 % or more homologous with the above-mentioned sequence of sonic hedgehog protein.

The sonic hedgehog precursor protein is composed of the amino acids 1 - 462 of the sequence described in the EMBL database under No. L38518. The amino acids 1 - 23 represent the signal peptide, the amino acids 24 - 197 represent the mature signal domain, the amino acids 32 - 197 represent the signal domain shortened by 8 amino acids and the amino acids 198 - 462 represent the auto-processing domain after autoproteolytic cleavage.

The first 8 amino acids of the hedgehog protein are understood by the invention as the first 8 amino acids of the processed protein for example the amino acids 24-31 for sonic hedgehog protein.

Surprisingly when hedgehog proteins are produced recombinantly in the baculovirus expression system, a highly active mutant of the protein (activity increased by at least 10-fold, preferably at least 100-fold compared to shh) accumulates in the initial period of the fermentation. This mutant of the polypeptide according to the invention can be in particular isolated when the fermentation is terminated at the latest after ca. 30 hours, preferably after ca. 24 - 27 hours. This is also surprising since a fermentation period after infection of at least 2 days has been previously described for the production of hh proteins in the baculovirus expression system (Bumcrot et al., Mol. Cell. Biol. (1995) 2294 - 2303). It has also been described for other proteins which are produced in the baculovirus system such as rhodopsin kinase (Cha et al., Proc. Natl. Acad. Sci. USA 94 (1997) 10577 - 10582) that a maximum of protein and activity is achieved after 64 - 88 h. According to the invention it was found for hedgehog proteins that although the amount of hedgehog protein in the fermentation supernatant greatly increases in the period between 33 and 72 hours, mainly hh protein with an activity that is known from the prior art is formed in this period. In contrast the amount of such a hh protein is considerably less (at least 5 - 10-fold) when the fermentation period is reduced to below ca. 30 h. which allows the identification and isolation of the highly active hh protein mutant according to the invention.

The hh mutant according to the invention is very sensitive towards proteases which is why it is preferable to add protease inhibitors such as for example aprotinin, PMSF or pepstatin or a mixture thereof to the supernatant of the fermentation.

Furthermore it is preferable to add non-ionic detergents such as polysorbate (e.g. Triton®X100) during the purification, preferably at least after the first crude purification over heparin-Sepharose, since this also stabilizes the hh proteins according to the invention.

In a first step for the purification of the protein according to the invention it is expedient to carry out a chromatography on heparin-Sepharose. It is preferable to carry out this chromatography as a step elution i.e. preferably to elute at a concentration of at least 0.7 mol/l (preferably 1.2 mol/l) after washing with 250 mmol/l NaCl.

It is particularly preferable to carry out a hydroxylapatite chromatography to purify the hh mutant according to the invention. This achieves a good concentration of the activity with relatively low losses (< 50 %). Further suitable chromatographic steps are for example a heparin-Sepharose chromatography (Miao et al., J. Neurosci. 17 (1997) 5891 - 5899) which is, however, preferably carried out in the presence of non-ionic detergents. Furthermore it is preferable to carry out a dialysis after the heparin-Sepharose chromatography preferably against low ionic strengths (e.g. buffer containing 1 - 10 mmol/l sodium phosphate pH 6.5 - 7.5). It is particularly preferable that in this dialysis the buffer against which it is dialysed contains 10 - 100 mmol/l, preferably 50 mmol/l sodium chloride and that the dialysis is carried out at a low concentration of the hh protein (1 mg/ml or less, preferably 0.5 mg/ml or less).

Furthermore it is also preferable to add suramin during the purification or at least before determining the activity of the protein. This also stabilizes the activity. In the case of suramin it was previously only known that it is suitable for detaching hh proteins from the cell surface or the extracellular matrix (Bumcrot et al., see above).

For the further purification it is preferable to again chromatograph on heparin-Sepharose and hydroxylapatite.

In a further embodiment of the invention the hh mutant according to the invention can be used to produce a pharmaceutical composition which is also a subject matter of the invention. This pharmaceutical composition contains a pharmacologically effective dose of the protein according to the invention and can be administered systemically as well as locally. It is also preferable to use the proteins according to the invention in combination with other proteins of the hedgehog family or bone growth factors such as bone morphogenetic proteins (BMPs), (Wozney et al., Cell. Mol. Biol. of Bone, Bone Morphogenetic Proteins and their Gene Expression, 131 - 167, Academic Press Inc. 1993) or parathyroid hormones (Karablis et al., Genes and Development 8 (1994) 277 - 289).

The protein according to the invention can be used advantageously to induce chondrocytes and osteocytes in an osteoinductive pharmaceutical composition. Osteoinductive pharmaceutical compositions are for example known from the US patent 5,364,839, WO 97/35607, WO 95/16035.

When the protein according to the invention is administered locally it is preferable to use it in combination with a suitable matrix as a carrier and/or with a sequestering agent. Such a matrix is suitable for slowly releasing the protein in vivo in an active form in particular in the vicinity of bones. The sequestering agent is a substance which facilitates administration for example by injection and/or prevents or at least delays migration of the protein according to the invention from the site of administration.

A biocompatible degradable material for example based on collagen or other polymers based on polylactic acid, polyglycolic acid or co-polymers of lactic acid and glycolic acid are particularly suitable as a matrix material. Such polymer matrices are described for example in WO 93/00050.

Sequestering agents are for example cellulose and cellulose-like materials and for example alkyl cellulose, carboxymethyl cellulose, hyaluronic acid, sodium alginate, polyethylene glycol and polyvenyl alcohol of which hyaluronic acid is particularly preferred especially in a pharmaceutical composition even without carrier matrix.

It is also preferable for the production of the pharmaceutical composition to add auxiliary substances such as mannitol, sucrose, lactose, glucose or glycine and antioxidants such as EDTA, citrate and detergents, preferably non-ionic detergents like polysorbates and polyoxyethylenes.

In a further preferred embodiment a pharmaceutical composition of the hedgehog protein according to the invention together with suramin is preferred and can be advantageously used.

The following examples, publications and figures further elucidate the invention, the protective scope of which results from the patent claims. The described methods are to be understood as examples which still describe the subject matter of the invention even after modifications.

Description of the Figures:

Fig. 1: Kinetics of the secretion of alkaline phosphatase inducing activity (bars) and hh mutant (dots and line) by high five cells after infection with baculovirus.

Fig. 2: Elution diagram of the purification of the fermentation supernatant with heparin-Sepharose

Fig. 3: Elution diagram of the purification of the dialysed eluate of the heparin-Sepharose with hydroxylapatite

Fig. 4: Elution diagram of the purification of the dialysed active fractions of the hydroxylapatite column with a 1 ml HiTrap heparin column.

Fig. 5: Alkaline phosphatase inducing activity of the fractions of the 1 ml High Trap heparin chromatography.

Fig. 6: Coomassie staining of SDS-PAGE with alkylated fractions of the 1 ml High Trap heparin chromatography.

Fig. 7: Western blot with an antibody against the N-terminus of shh of the SDS-PAGE with alkylated samples of the fractions of the 1 ml High Trap heparin chromatography.

Fig. 8: Western blot with an antibody against the N-terminus of shh of the SDS-PAGE with reduced samples of the fractions of the 1 ml High Trap heparin chromatography.

Fig. 9: Activity of the retained and permeated material after filtration of active shh fractions using membranes with an exclusion limit of 30 and 100 KDa

Fig. 10: Influence of suramin on the activity of the hh mutant: No suramin (B), suramin ad 0.1 mg/ml only added after dialysis against PBS+0.05 % Tween®80 (C) or suramin ad 0.1 mg/ml added before dialysis and dialysed against PBS+0.05 % Tween®80 containing additionally 0.1 mg/ml suramin were added to aliquots of an active fraction after hydroxylapatite chromatography.

Fig. 11: Influence of Tween®20 and Tween®80 on the activity of the hh mutant: Aliquots of a pool of AP active fractions after SP Sepharose chromatography in 50 mM NaPi, 0.9 M NaCl, 1 mM EDTA pH 7.3 were admixed with the stated concentrations of Tween and dialysed against PBS

containing the respective concentration of Tween. The samples were sterile filtered through 0.2 µm filters before being used in the C3H10T1/2 test.

Fig. 12: Influence of trypsin and chymotrypsin on the activity of the hh mutant: AP active fractions after a step elution of heparin-Sepharose were adjusted to a protein concentration of 0.46 mg/ml in 10 mM Na phosphate, 0.05 % Tween®80 and admixed with trypsin or chymotrypsin at a protease/protein ratio (w/w) of 1:100 (A), 1:500 (B), 1:2500 (C) and 1:10000 (D). The samples were incubated for 11 h at RT. The digestion was stopped by adding aprotinin in a 5-fold weight excess and the samples were analysed in SDS-PAGE (A:) and in the C3H10T1/2 test (B:). 1, test mixture; 2, control without protease; 3, samples treated with trypsin; 4, samples treated with chymotrypsin; 5, control trypsin (1:100) and aprotinin at t=0; 6, control chymotrypsin (1:100) and aprotinin at t=0.

Example 1

Expression of recombinant human sonic hh (shh)

The N-terminal domain of human shh with the amino acids 24 - 197 (EMBL accession No. L 38518) was as described by Miao (J. Neurosci. (1997) 17, 5891 - 5899) and Bumcrot et al., (Mol. Cell. Biol. (1995) 15, 2294 - 2303) for the rat protein by means of recombinant baculovirus in High five cells (Invitrogen, Leek, NL, Order No. E 855-02) using Excell 400 medium (JHR, Inc.) in which sufficient virus was used to infect each cell on average with one virus (multiplicity of infection (m.o.i.):1).

The fermenter contents were clarified after 26 or 72 h by centrifugation at 1000 g and filtration and the supernatant or the permeate was stored at -80°C until further use. Fermentation samples were analysed for their content of alkaline phosphatase inducing activity [Nakamura et al. (1997), Kinto et al. (1997) FEBS Lett. 404, 319-323] and for their content of shh protein by means of RP-HPLC (Vydac C18, gradient of 0-90 % acetonitrile in 0.1 % trifluoroacetic acid, TFA) or SDS-PAGE.

The fermentation was terminated after 24 - 32 h (preferably after 24 - 27 h) fermentation time and the supernatant was clarified.

Example 2

Purification of the active hh mutant

1 Tablet of "complete" inhibitor mix (Boehringer Mannheim GmbH, order No. 1873580) was added per 50 ml supernatant to the clarified supernatant after thawing and 3.5 l of this solution was applied at 4°C to a heparin-Sepharose column (volume 90 ml; Pharmacia Biotech) which had previously been equilibrated with 20 mM sodium phosphate, pH 7.2. After the sample application it was washed with 20 mM sodium phosphate, 0.05 % Tween®80, pH 7.2 (= buffer A) and unspecifically bound protein was eluted by a wash step with buffer A which additionally contained 0.25 M NaCl. The activity was obtained by a subsequent elution with buffer A which additionally contained 1.2 M NaCl.

This eluate was subsequently diluted with one volume 10 mM sodium phosphate, 0.05 % Tween®80, 50 mM NaCl, pH 7.2 (= buffer B) and dialysed against buffer B at 4°C.

The dialysate was applied to a hydroxyapatite column (volume 10 ml; Makro Prep; 40 µm, type I; BIO-Rad) equilibrated with buffer B. It was eluted with a gradient of 10 to 300 mM NaP in buffer B (2 x 200 ml).

Aliquots of the fractions were analysed for their ability to stimulate alkaline phosphatase in a mouse fibroblast cell line e.g. C3H10T1/2 cells as well as by means of SDS-PAGE and RP-HPLC. The remainder of the fractions was stored at -80°C until further processing. The maximum activity elutes at the end of the gradient between 0.25 - 0.3 M sodium phosphate whereas inactive or only weakly active forms of shh already elute much earlier from the column.

The active fractions were pooled and dialysed against buffer B at -4°C and applied to a 1 ml HiTrap heparin column (Pharmacia Biotech) which had been equilibrated with 20 mM potassium phosphate, 0.05 Tween®80, pH 7.2. It was eluted by a gradient of 0 - 1400 mM KCl in 20 mM potassium phosphate, 0.05 % Tween®80, 50 mM NaCl, pH 7.2. Active fractions were identified by the stimulation of alkaline phosphatase in C3H10T1/2 cells, and alkylated and reduced samples were analysed by means of SDS-PAGE and Western blot with an antibody against the N-terminus of shh.

In contrast to inactive fractions, the active fractions contain a hh mutant which under alkylating conditions exhibits a molecular weight which is 1 - 3 kDa lower than only weakly active shh with an intact N terminus. However such a difference in molecular weight is not detectable under reducing conditions.

Example 3

Induction of alkaline phosphatase in the cell test (determination of the activity of alkaline phosphatase)

5000 cells of the murine mesenchymal pluripotent line C3H10T1/2 (ATCC CCL-226) were sown in each well of a 96-well microtitre plate. The cells were in 100 µl DMEM, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 % foetal calf serum, FCS. On the next day the active substances to be examined were added at the appropriate concentrations in a volume of 100 µl. The test was stopped after 5 days. For this purpose the supernatants were discarded and the cells were washed once with PBS. The cells were lysed in 50 µl 0.1 % Triton®X-100 and frozen at -20°C. After thawing 25 µl was used for the protein determination and 25 µl for the determination of the activity of alkaline phosphatase.

Protein determination according to the instructions of the manufacturer Pierce:
75 µl redistilled H_2O was added to the mixture, then 100 µl BCA protein reagent was added (Pierce Micro BCA, No. 23225). After 60 min the optical density (OD) at 550 nm was measured.

Activity of the alkaline phosphatase according to the instructions of the manufacturer Sigma:
100 µl reaction buffer (Sigma 221) was added to the preparation. A substrate capsule (Sigma 104-40) was dissolved in 10 ml redistilled H_2O and then 100 µl was added to the test mixture by pipette. The OD was measured at 405 nm after the yellow coloration. In the reaction alkaline phosphatase converts p-nitrophenyl phosphate into p-nitrophenol.

The ODs were each converted into nmol or µg by means of standard curves. The evaluation was according to the formula:

nmol PNP per (measured) minute per mg (cell) protein

Results:

- Shh (monomer) from E. coli at a concentration of 43 µg/ml (=2.15 µmol/l) in the cell test results in 8.537 nmol pNP/min/mg
  Shh (dimer) from E. coli at a concentration of 41.5 µg/ml (=1.037 µmol/l) in the cell test results in 5.133 nmol pNP/min/mg
- hh mutant according to the invention at a concentration of 0.1 µg/ml (=5 nmol/l) with 0.1 mg/ml suramin in the cell test results in 88.762 nmol pNP/min/mg
- hh mutant according to the invention at a concentration of 0.1 µg/ml (=5 nmol/l) without suramin in the cell test results in 44.828 nmol pNP/min/mg
- a control without shh results in 1.292 nmol pNP/min/mg
- BVCM used in a 1/40 dilution results in 41.961 nmol pNP/min/mg (=internal positive control)

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